



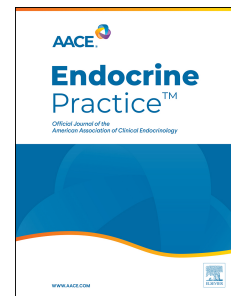
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Treatment with 25-hydroxyvitamin D3 (calcifediol) is associated with a reduction in the blood neutrophil-to-lymphocyte ratio marker of disease severity in patients hospitalized with COVID-19: a pilot, multicenter, randomized, placebo-controlled double blind clinical trial

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PII: S1530-891X(21)01259-3

DOI: <https://doi.org/10.1016/j.eprac.2021.09.016>

Reference: EPAC 274

To appear in: *Endocrine Practice*

Received Date: 3 June 2021

Revised Date: 15 September 2021

Accepted Date: 15 September 2021

Please cite this article as: Maghbooli Z, Sahraian MA, Jamali-Moghadam SR, Asadi A, Azadeh zarei MD, Zندهdel A, Varzandi T, Mohammadnabi S, Alijani N, Karimi M, Shirvani A, Holick MF, Treatment with 25-hydroxyvitamin D3 (calcifediol) is associated with a reduction in the blood neutrophil-to-lymphocyte ratio marker of disease severity in patients hospitalized with COVID-19: a pilot, multicenter, randomized, placebo-controlled double blind clinical trial, *Endocrine Practice* (2021), doi: <https://doi.org/10.1016/j.eprac.2021.09.016>.

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Title:

Treatment with 25-hydroxyvitamin D₃ (calcifediol) is associated with a reduction in the blood neutrophil-to-lymphocyte ratio marker of disease severity in patients hospitalized with COVID-19: a pilot, multicenter, randomized, placebo-controlled double blind clinical trial

Abbreviated title:

Therapeutic effects of oral 25-hydroxyvitamin D₃ on COVID-19

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Abstract

Objective: The goal of this randomized placebo-controlled clinical trial was to investigate the therapeutic efficacy of oral 25-hydroxyvitamin D₃ [25(OH)D₃] in improving vitamin D status in vitamin D deficient/insufficient patients infected with the SARS-CoV-2 (COVID-19) virus.

Methods: This is a multicenter randomized double blinded randomized placebo-controlled clinical trial. Participants were recruited from three hospitals that are affiliated to [Institution Blinded for Review], and [Institution Blinded for Review].

Results: A total 106 hospitalized patients who had a circulating concentration of 25(OH)D <30 ng/ml were enrolled in this study. Within 30 and 60 days 79.4% (26 out of 34) and 100% (24 out of 24) of the patients who received 25(OH)D₃ became sufficient whereas ≤12.5% the patients in the placebo group became sufficient during 2 months follow-up.

We observed an overall lower trend for hospitalization, ICU duration, needing ventilator assistance and mortality in the 25(OH)D₃ group compared with placebo group but they weren't statistically significant. Treatment with oral 25(OH)D₃ was associated with a significant increase in the lymphocyte percentage and decrease in the ratio of neutrophils to lymphocytes (NLR) in the patients. The lower NLR was significant associated with reduced ICU admission days and mortality.

Conclusion: Our analysis indicated that oral 25-hydroxyvitamin D₃ was able to correct vitamin D deficiency/insufficiency in COVID-19 patients that resulted in improved immune function by increasing blood lymphocyte percentage. RCTs with a larger sample size and with higher dose of 25(OH)D₃ maybe needed to confirm the potential effect of 25(OH)D₃ on reducing clinical outcomes in COVID-19 patients.

Ethics and Dissemination: The study protocol was approved by the Ethics Committee of [Institution Blinded for Review]. (Approval Number: IR.TUMS.VCR.REC.1399.061). Dissemination plans include academic publications, conference presentations and social media.

Trial registration:

The protocol was registered with the Iranian

Registry of Clinical Trials (IRCT) on April 11, 2020 [Number Blinded for Review]. and U.S. National Institutes of Health [Number Blinded for Review] on May 11, 2020.

Keywords: COVID-19, 25-hydroxyvitamin D₃, viral infection, supplementation, lymphocyte, vitamin D deficiency

Introduction:

Coronavirus disease 2019 (COVID-19) is a respiratory and systemic disorder caused by the coronavirus 2 (SARS-CoV-2). This pandemic has caused as of May 13, 2021 a total of 139,933,765 confirmed cases and 3,346,652 deaths in the world (1). Higher mortality and morbidity rates have been observed in patients with severe pneumonia and in some case associated with multi-organ failure (2) and occurs in approximately half of hospitalized patients (3). A recent large meta-analysis on more than 10,000 subjects demonstrated that vitamin D supplementation had a protective role in acute respiratory infections, in adults (4). Due to these findings, an important role for vitamin D has been suggested in the treatment or prevention of COVID-19.

Recently, observational studies have reported a link between vitamin D deficiency and morbidity and mortality associated with COVID-19 (5-7). But few trials have been conducted to determine if improvement in vitamin D status during hospitalization provided any benefit (8, 9). The most common form of dietary vitamin D supplementation used today is cholecalciferol or vitamin D₃. It is hypothesized that increasing serum 25(OH)D₃ levels above 30 ng/ml and in the range of 40-60 ng/ml may noticeably reduce the severity and mortality of various viral diseases, including COVID-19 (10-12). The Endocrine Society guidelines recommends for adults that 1500-2000 IU/d of vitamin D may be required to raise the blood level of 25(OH)D consistently above 30 ng/ml (75 nmol/liter) (13). It takes at least 3-4 weeks of 1000 IU/d of vitamin D₃ to reach a plateau in the range of 30 ng/ml in circulating serum concentrations of 25(OH)D₃ (14, 15).

As an alternative strategy to raise serum 25(OH)D₃ in vitamin D-deficient adults, oral supplementation of 25-hydroxyvitamin D₃ (calcifediol) has been suggested (15). When vitamin D₃ is ingested, it gets incorporated into chylomicrons and enters the lymphatic system. The chylomicrons then enter into the bloodstream via the superior cava. Most of the vitamin D is incorporated into the body fat. Vitamin D₃ in the circulation and the vitamin D₃ that is slowly released from the body fat into the circulation is converted in the liver to 25(OH)D₃. This is the likely explanation for why it takes approximately 3-4 weeks to achieve a steady state concentration of 25(OH)D₃ (14, 16, 17). 25(OH)D₃ is more hydrophilic and therefore after its ingestion is absorbed into the venous portal system thereby rapidly increasing circulating concentrations of 25(OH)D₃. It was reported that orally administered 20 µg 25(OH)D₃ compared to 800 IU (20 µg) vitamin D₃ was significantly more efficient and rapid in raising serum concentrations of 25(OH)D₃ in healthy postmenopausal women into a desirable range of at least 30 ng/mL. The rapid increase in serum concentrations of 25(OH)D₃ was related to a decrease in innate immunity markers including eotaxin, IL-12, MCP-1, and MIP-1 β (16). After oral consumption of 25(OH)D₃, the major circulating form of vitamin D, it is converted in the kidneys to 1,25-dihydroxyvitamin D [1,25(OH)₂D] through CYP27B1 (1-α-hydroxylase) and enters circulation and interacts with vitamin D receptor (VDR) for the purpose of regulating calcium and bone metabolism (18, 19). The activated monocytes and macrophages express CYP27B1, producing 1,25(OH)₂D from circulating 25(OH)D₃, inducing antibacterial agents (18, 19).

Thus, 25(OH)D₃ consumption would be improve vitamin D status more rapidly and be more available for target immune cells for fighting with coronavirus. We aimed to investigate the potential therapeutic benefit of rapidly increasing circulating serum 25(OH)D₃ concentrations with orally administered 25(OH)D₃ in patients with COVID-19.

Material and methods:

Study design and participants

This multicenter clinical trial was designed as a randomized double blinded placebo controlled. Participants were recruited from hospitals that are affiliated to [Blinded] University of Medical Sciences (TUMS) ([Institutions Blinded] hospitals), and [Second Blinded] University of Medical Sciences ([Institution Blinded] hospital).

The recruitment was started on May 2020, and the study run until October 2020. All measurements were analyzed at the admission date, release date, and after first- and second month follow-up.

COVID-19 (SARA-Cov-2) was diagnosed by acute respiratory tract infection symptoms (e.g. fever, cough, and dyspnea) with no other etiology that fully explained the clinical presentation. The diagnosis was supported by chest computed tomography (CT) scan findings compatible with Covid-19 and/or a definitive diagnosis of Covid-19 with real-time polymerase chain reaction (PCR).

Inclusion and exclusion criteria

Inclusion criteria of study subjects were as follows:

1. Older than 18 year-old
2. No medications or disorders that would affect vitamin D metabolism
3. Vitamin D deficiency/insufficiency (25(OH)D < 30 ng/mL).
4. Ability and willingness to give informed consent and comply with protocol requirements

Exclusion criteria were as follows:

1. Pregnant or lactating women.
2. Severe underlying diseases, such as advanced malignant tumor, end-stage lung disease, etc.
3. Chronic hepatic dysfunction, intestinal malabsorption syndromes including inflammatory bowel disease.
4. Ongoing treatment with pharmacologic doses of vitamin D, vitamin D metabolites or analogues
5. Supplementation with over the counter formulations of vitamin D₂ or vitamin D₃

6. Use of tanning bed or artificial UV exposure within the last two weeks.
7. Consuming medication affecting vitamin D metabolism or absorption (anticonvulsants, anti-tuberculosis medication glucocorticoids, HIV medications and cholestyramine).
8. History of an adverse reaction to orally administered vitamin D, vitamin D metabolites or analogues.
9. History of elevated serum calcium >10.6 mg/dl; that was corrected for albumin concentration or subjects with a history of hypercalciuria and kidney stones.
10. History of conditions that could lead to high serum calcium levels such as sarcoidosis, tuberculosis, and some lymphomas associated with activated macrophages which increase the production of 1,25(OH)₂D.
11. Inability to give informed consent.

Recruitments and inform consent process

Eligible subjects were enrolled in the study after consenting process to provide a blood sample to evaluate serum 25(OH)D₃ level. All participant with vitamin D deficient/insufficient (25(OH)D₃ < 30 ng/mL) were randomized (13). The study flow diagram is shown as figure 1.

Once the subjects were determined to be eligible, they were presented with the consent form by trained research nurses. The participants also received information sheets. Research Nurses were discussing the trial with subjects in light of the information sheets. Also, there were a plan to provide medical advice or counseling to subjects who were screened and met the 25(OH)D₃ < 30 ng/mL criteria who decided not to participate in the study.

Randomization

All participants in a stratified random sampling method were recruited in the 25(OH)D₃ or placebo group with a ratio of 1:1. The clinical coordinator determined this with a computer-generated randomization program. Subjects in treatment group (n=53) received 25(OH)D₃ and non-treatment group (n=53) received placebo. The randomization time was at the day of admission to take oral 25(OH)D₃ or placebo.

Intervention

The 25(OH)D₃ and placebo capsules were generously provided by Carbogen-Amcis BV, a company belonging to the Dishman Group (Ahmedabad, India), 25(OH)D₃ was formulated in median chain fatty acids and then encapsulated. The placebo contained the same amount of medium chain fatty acids and was also encapsulated. The participants received randomly either a bottle containing 30 capsules of 25(OH)D₃ or placebo in their first visit and then again 30 days later. The bottles were returned to be counted at each visit. The dose of 25(OH)D₃ was 25 µg administered orally once daily. At the time that we initiated our clinical research trial, there was no evidence to suggest that a higher dose of vitamin D₃ or 25-hydroxyvitamin D₃ would be more effective in reducing risk for morbidity and mortality in COVID-19. Because of safety concerns, we used a dose of 25(OH)D₃ that was equivalent to approximately 3000-6000 IU per day vitamin D₃. The study was suspended if the serum calcium was consistently above the normal range or serum 25(OH)D₃ was above 100 ng/ml. During trial, there were not any patients with serum calcium levels >10.6 mg/dl or serum 25(OH)D > 100 ng/ml. There were not any adverse reactions reported by participants during consumption of oral 25(OH)D₃ or the placebo.

Blinding

All subjects at the clinical departments were blinded to trial intervention allocation. The main outcomes were evaluated by physicians.

Compliance

The subjects were followed up weekly by phone to remind study participants to use their study medication and to monitor dosing compliance as well as to ask about their medical symptoms. The subjects were asked to return the first and second bottles of study medication after first- and second-month of hospital admission date; respectively, for re-counting to evaluate their compliance and assessing biochemical tests as well as serum concentrations of 25(OH)D₃.

Study Outcomes

1. Severity of COVID-19 (SARS-Cov-2) infection: Percentage of mild, moderate and severe forms of COVID-19 based on WHO criteria

2. Length of stay in hospital: days from admission to discharge from hospital

3. Oxygen support: percentage of COVID patients who need oxygen support

4. Death: rate of death due to COVID-19 during the study

5. Lymphocyte count and percentage

6. Serum concentrations of 25(OH)D at baseline and after 30 and 60 days of starting oral 25(OH)D₃ or placebo (first month and second month follow-up)

Study measurements

Data included following information: demographic information (age, sex, body mass index (BMI)), smoking habit, medical history, principal clinical symptoms and their onset time, RT-PCR results, radiological findings, laboratory findings, comorbidities, and disease progression.

Laboratory examination at the time of admission to the hospital or soon thereafter included a complete blood count, blood biochemistries (total 25(OH)D, calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), alanine transaminase (ALT), aspartate aminotransferase (AST), Creatine kinase (CK), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), C-reactive protein (CRP), procalcitonin (PCT), erythrocyte sedimentation rate (ESR), bilirubin (mg/dl), and also arterial blood gas (PO₂, PCO₂, HCO₃, pH). Total serum 25(OH)D was measured by HPLC. The method of HPLC was described in our recent study (20).

Statistical analysis

Data were analyzed by SPSS statistical software (version 20). Continue variables were presented as mean (standard deviation [SD]) for with normally distributed or median (interquartile range [IQR]) for non-normally distributed data. Parametric and non-parametric tests including the independent t test, Mann-Whitney U test, were used to compare differences between continue variables where appropriate. The categorical variables were presented as percentage and chi square or Fisher's exact test was applied to examine the percentage differences of the sign and symptom, requiring mechanical ventilation, and requires intensive care and hospital mortality rates in treated and placebo groups. The standardized mean difference (SMD) was used to express the size of the intervention effect on raising circulating vitamin D

levels in the treatment group compared with the placebo group. Logistic regression model was used to consider independent association of the neutrophil-to-lymphocyte ratio (NLR) and clinical outcomes.

All tests were two-sided, and P values <0.05 were considered significant.

Safety

Since the clinical trial was designed as minimal risk a formal committee for data monitoring was not required. However, potential toxicity was monitored at two steps; first- and second month follow-up, for a serum 25(OH)D₃, calcium, albumin and creatinine. We monitored for early signs and symptoms of vitamin D toxicity and hypercalcemia in all participants. The subjects were followed up weekly by phone to ask about their medical symptoms.

Ethics and Dissemination

The Declaration of Helsinki fully considered during this clinical trial. The Ethics Committee of the [Blinded] University of Medical Sciences approved this clinical trial (Approval Number: IR.TUMS.VCR.REC.1399.061). A SPIRIT checklist is available for this protocol. This clinical trial has been registered at ClinicalTrials.gov with the identifier [Number Blinded for Review]. Participants signed informed consent.

Results:

Based on inclusion criteria, a total 106 vitamin D deficient/insufficient hospitalized patients were enrolled in this study: 53 placebo group, 53 treatment group with 25(OH)D₃ (figure 1).

All patients received the same standard care; a combination of hydroxychloroquine, azithromycin and for patients with pneumonia ceftriaxone was used. During hospitalization, all participants received 30 capsules (first box) 25(OH)D₃ or placebo to take in hospital and continued at home if they were discharged earlier. After the 30 days of starting 25(OH)D₃ or placebo, all participants who were released from the hospital and who visited the outpatient COVID-19 centers were recruited to take the second box of capsules; 38 of treatment group and 31 of placebo group. For second month of follow-up, 24 of treatment group and 19 of placebo group returned to the outpatient COVID-19 centers. Potential toxicity

was monitored in each follow-up visit (after 30 days and 60 days of starting 25(OH)D₃ or placebo) for a serum concentrations of calcium, albumin, creatinine (Table S1) and 25(OH)D. Concern about COVID-19 reinfection was the main reason of lost to follow-up.

The Baseline and clinical characteristics

The baseline and clinical characteristics of the included participants are summarized in table 1. The mean age of all participants was 49.1±14.1 years; 48.9±13.6 in men and 49.5±14.9 in women. There were no significant age and sex differences in each group. There were not significant differences in hematologic, and biochemical tests and serological markers (table 1).

The severity of disease considered based on CDC criteria; dyspnea, respiratory frequency ≥30/minute, blood oxygen saturation < 93%, and/or lung infiltrates >50% of the lung field within 24-48 hours.

At time of admission (baseline), the severity of COVID-19 was observed similar in both groups (67.9% placebo group and 60.4% in 25(OH)D₃ group, p=0.41). In term of prognostic factors of COVID-19, there were no significant differences between two groups (table 2).

Improvement of circulating serum levels of 25(OH)D

Figure 2 shows the 25(OH)D₃ concentrations at baseline, after first- and second month follow-up. After 30 days of using 25(OH)D₃ or placebo, circulating concentrations of 25(OH)D₃ was significantly increased in the patients who received 25(OH)D₃ compared to the placebo group (42.0±13.7 ng/ml, vs. placebo: 19.3±8.5 ng/ml) (figure 2). The delta serum concentrations of 25(OH)D₃ were 23.6±10.4 ng/ml in treatment group compared with 0.8±4.2 ng/ml in placebo group; 79.4% of treatment group and 12.5% of placebo group had circulating 25(OH)D₃ concentrations greater than 30ng/ml.

After 60 days of using oral 25(OH)D₃ or placebo, circulating concentrations of 25(OH)D₃ were significantly increased in the patients who receive 25(OH)D₃ compared with placebo group (treatment group: 59.6±18.6 ng/ml, vs. placebo: 19.4±7.0 ng/ml). The delta serum levels of 25(OH)D₃ were 40.02±19.2 ng/ml in treatment group compared with 1.4±6.5 ng/ml in placebo group; all patients in the treatment group and 10.5% of placebo group had circulating 25(OH)D₃ levels higher than 30ng/ml.

Standardized Mean Difference (SMD) was used to express the size of the intervention effect on raising circulating vitamin D levels in the treatment group compared with the placebo group. The treatment group had 1.92 effect size (standardized mean difference (SMD)= 1.92, 95% CI: 1.38, 2.45) on increased circulating 25(OH)D₃ concentrations after the first month follow-up and 2.73 effect size (SMD= 2.73, 95% CI: 2.11, 3.35) on increased circulating 25(OH)D₃ concentrations after the second month of follow-up.

The compliance of taking capsules (25OHD₃ or placebo) was; 89% in treatment group (95% CI: 85, 94) vs. 93% in placebo group (95% CI: 90, 96).

COVID clinical features

There was an overall trend for lower hospitalization duration in the 25(OH)D₃ group compared with placebo group that was not statistically significant (Median (IQR): 5 (3) vs. 6 (5.5), p=0.1).

Among treatment group 6 patients were admitted in ICU compared to 10 patients in placebo group. Also, 2 patients in treatment group and 5 patients in placebo group needed ventilator. Death occurred in 3 patients in treatment group compared to 5 patients in placebo group. There were no statistically significant differences in ICU admissions, need for ventilation and rate of death, between patients receiving 25(OH)D₃ compared to those receiving the placebo (Table 3).

During hospitalization, all patients were treated with hydroxychloroquine and antibiotics (Azithromycin or Ceftriaxone). There was no significant difference between-group in the proportion of patients treated with corticosteroids (less than 10 mg/day dexamethasone, or equal/less than 25mg methyl-prednisolone), or antiviral drugs (interferon). Also, in the regression model antiviral or glucocorticoid treatment had no significant effect on the NLR and no effect on the relationship between 25(OH)D₃ treatment and decreasing the NLR. The biochemical tests at release time are presented in table S2. To consider the effect of oral consumption of 25(OH)D₃ during hospitalization, the mean differences of all biochemical and hematological tests were calculated; at base line and at that time that they were released from hospital.

Patients who have received 25(OH)D₃ had a significant increase in the percentage of lymphocytes ($p=0.03$). In 25(OH)D₃ group, the neutrophil-to-lymphocyte ratio (NLR) was less than placebo group (SMD= -0.81, 95% CI: -1.21, -0.41). After the patients were released from the hospital, they had a follow-up visit 1 and 2 months later at which time the NLR in their circulation was determined. The circulating NLR decreased in both groups and were statistically no different (Figure 3).

There was no significant difference in mean changes of platelet number and serum concentrations of LDH between two groups ($p=0.96$, $p=0.57$, respectively).

The data analysis showed that there were not any significant differences in re-admission. There was not any mortality in two groups after first- and second- month of follow up.

Regarding to the modifier role of 25(OH)D₃ on treatment, the relationship of NLR was considered with main outcomes of COVID-19 including ICU admission days, and mortality.

There was significant correlation between lower NLR and reduced ICU admission days and the NLR ($\rho=0.3$, $p=0.004$). In a logistic regression model, after adjusting age, sex, BMI and history of chronic disorders, there was independent association between NLR at the time of discharge and needing ICU admission ($p=0.002$, OR=1.2).

Although in our study population a few patients died, the NLR at the last day of hospitalization was about 4 times higher than patients who survived (median (IQR); 15.7(7.7) vs. 3.1(5.1)).

Among biochemical outcomes, there was significant correlation between the NLR and LDH ($p=0.02$, $r=0.3$). However, after adjusting age, sex and history of chronic disorders there was no significant association between the NLR and LDH at the time of discharge ($p=0.08$).

Discussion:

To assess the therapeutic effect of rapidly improving vitamin D status in hospitalized patients with COVID-19, we designed a randomized double blinded controlled clinical trial whereby hospitalized patients received either 25µg of 25(OH)D₃ (calcifediol) or placebo daily and continued for 60 days.

In our trial, the rationale was to give 25(OH)D₃ to the COVID-19 patients to increase and sustain circulating blood concentrations of 25(OH)D₃ and relate this effect to clinical outcomes. One of the advantages of using 25(OH)D₃ is that serum 25(OH)D increases more rapidly than using vitamin D₃ since conversion to 25(OH)D is not required (21). Therefore, oral 25(OH)D₃ is able to correct vitamin D deficiency more rapidly and consistently than oral vitamin D₃ (21, 22).

Based on vitamin D biological potency, one IU of vitamin D₃ is equivalence with 0.025 µg; proportionally, 25 µg = 1000 IU vitamin D₃ (23). However, there is not a gold standard for the equivalence between IUs and molecular mass with 25(OH)D₃.

Clinical trials have demonstrated that oral 25(OH)D₃ was 3-6 times more effective in rapidly raising circulating levels of 25(OH)D₃ compared to oral vitamin D₃ on a weight basis (22). Therefore, 25 µg of 25(OH)D₃ is not equivalent to 25µg (1000 IU) of vitamin D₃. It would be equivalent to 75-150 µg or 3000-6000 IU of vitamin D₃. Barger-Lux et al. evaluated different dosages of oral 25(OH)D₃ (calcifediol) compared to vitamin D₃ (24). They showed that “when using dosages ≤ 25 µg/day, serum 25OHD increased by 1.5 ± 0.9 nmol/l for each 1 µg of vitamin D₃, whereas this was 4.8 ± 1.2 nmol/l for oral 25(OH)D₃ and the relative potency of 25(OH)D₃ to vitamin D₃ was 3 times higher. Also the authors stated the highest dose of 25(OH)D₃ (50µg/d), was 7–8-fold more potent than vitamin D₃ with similar dosages”.

Charoenngam et al. in a randomized placebo controlled crossover study investigated the pharmacokinetics of oral 25(OH)D₃ and oral vitamin D₃ in healthy and obese adults and in adult patients with fat malabsorption syndromes; a 900 µg single-dose of either vitamin D₃ or 25(OH)D₃. They observed that the blood levels of 25(OH)D₃ rapidly increased and reached a peak concentration within 8 hours whereas when the same healthy adult ingested this same amount of vitamin D₃ the serum concentrations of 25(OH)D₃ gradually increased and reached a maximum blood concentration within 48-72 hours (25). They also observed that obese and fat malabsorption patients who were unable to raise their circulating

concentrations of 25(OH)D₃ to a similar degree as healthy adults after ingesting vitamin D₃ were able to raise their circulating concentrations of 25(OH)D₃ to the same degree after ingesting 25(OH)D₃ as compared to the healthy adults(25).

In present trial, the mean baseline serum 25(OH)D₃ of all participates was at the range of 2-29 ng/ml. Our findings indicated that the size of the intervention effect on raising circulating 25(OH)D levels in the treatment group was significantly higher than the placebo group. Using daily 25 µg of 25(OH)D₃ in treatment group had 1.92 and 2.73 effect size on raised circulating 25(OH)D₃ levels compared with placebo group; after first- and second- month follow-up respectively. Our observation is consistent with the conclusion made by Quesada-Gomez and Bouillon who evaluated nine RCTs and concluded that “the conversion efficacy of oral 25(OH)D₃ would be 3.2-fold more effective when compared with the same dosages of oral vitamin D₃ and also, oral calcifediol is a linear dose-response curve, irrespective of baseline serum 25OHD, whereas the rise in serum 25OHD is lower after oral cholecalciferol, when baseline serum 25OHD is higher” (14).

As organ damage from the cytokine storm and proliferation of the SARS-CoV-2 virus progress rapidly soon after the infection and once the damage is done, it is difficult to reverse. Thus, the more rapid increase in serum concentrations of 25(OH)D₃ may provide an advantage reducing morbidity and mortality associated with infectious diseases like the coronavirus.

Raising total and free 25(OH)D concentrations could result in rapid entry into its target innate and adaptive immune cells resulting in the production of 1 α ,25(OH)₂D which interacts with the VDR to modulate immune function (16, 18, 26, 27). Therefore, using high dose of 25(OH)D₃ at time of hospital admission might help in treatment of COVID-19 by preventing the cytokine storm and subsequent ARDS which is commonly the cause of mortality.

Our data showed that patients who received oral 25(OH)D₃ demonstrated a statistically significant lower neutrophil-to-lymphocyte ratio (NLR) with -0.81 efficacy (compared to the placebo group. During an inflammatory response, leucocytes act as an innate immune response and lymphocytes are responsible for the specificity of adaptive immune response. They circulate in the blood and central lymphoid tissues and participate in a variety of host defense mechanisms against viral infections. These include “cell-mediated reactions against infected cells and particularly those involving cytotoxic T lymphocytes, co-operation in the induction of antibody responses, and the production of immune interferon” (28). Recent observational studies in COVID-19 patients revealed that most of the infected patients have higher leukocyte and lower lymphocyte counts (29). The NLR is also considered as an inflammation marker and a prognostic factor of systemic inflammation that is increased in COVID-19 patients with severe clinical consequences (30). In a recent meta-analysis by Lagunas- Rangel (31) the NLR values were found to increase in patients with severe COVID- 19 with 2.4 efficacy. Consistent with these studies, our data showed the NLR at the time of enrollment was a significant predictor of needing intensive care. Although, only a few patients died in our study, those patients who died had a NLR that was 4 times higher than the NLR at the time the patients were released from the hospital.

Based on these findings 25(OH)D₃ intervention was significantly associated with the decreased NLR in the hospitalized COVID-19 patients compared to the hospitalized patients who received the placebo. Although the decrease in the circulating NLR was associated with improved clinical outcomes, we could not conclude that the decrease in NLR was solely the effect of 25-hydroxyvitamin D₃ treatment and, therefore, that the increasing 25(OH)D concentration was responsible for the change in clinical outcomes. Some studies have reported that vitamin D deficiency is associated with high mortality and morbidity in COVID-19 patients. A recent meta-analysis on 27 observational studies identified that a positive association between vitamin D deficiency and the severity of the COVID-19 (OR = 1.64; 95% CI = 1.30-2.09). They also revealed that vitamin D insufficiency increase hospitalization (OR = 1.81, 95% CI = 1.41-2.21) and mortality from COVID-19 (OR = 1.82, 95% CI = 1.06-2.58) (7).

In a clinical trial conducted by Entrenas et. al. (9) COVID-19 patients who received high dose of oral 25(OH)D₃ reduced the need for ICU admission. These patients received 532 µg of oral 25(OH)D₃ on first day of admission and 266 µg of oral 25(OH)D₃ on days 3 and 7 of hospitalization, and then weekly until discharge or ICU admission (9). Similar to our study two retrospective cohort studies on patients admitted for COVID-19 (32, 33) reported treatment with calcifediol reduced the risk of requirement for critical care by more than 80% and reduced the mortality risk by more than 70%”(32).

In another clinical trial, Annweiler and et al. (34) considered the efficacy of bolus dose of 80000 IU vitamin D₃ supplementation taken during COVID-19 or in the preceding month in frail elderly. Survival rate was twofold higher compared to the control group; with adjusted hazard ratio equal 0.11 (95 %CI:0.03;0.48). They reported that “bolus vitamin D₃ supplementation during COVID-19 or in the preceding month was associated with less severe COVID-19 and better survival rate in elderly”.

Our data are consistent with these observations where we observed an overall lower trend for hospitalization and ICU duration, needing ventilator assistance and mortality in the 25(OH)D₃ group compared with placebo group. We couldn't show the significant improvement in the clinical outcomes in the patients who ingested 25(OH)D₃ compared to the control group, this could be due to the need to more rapidly improved serum 25(OH)D concentrations by giving a higher dose of 25(OH)D₃ as has been recently reported (9,34,35).

In our patient population, 50% of participants had at least a history of one chronic disorder. Based on epidemiology studies, about half of all patients suffering COVID-19 had a history of chronic disorders like hypertension, diabetes, cardiovascular disease, kidney disorder, cancers and immunological diseases (35) and mortality rate increased in the presence of serious comorbid medical conditions(35, 36). Based on various RCTs and meta-analyses, it has been observed that vitamin D supplementation, especially in deficient patients, can provide a clinically beneficial effect in some of these medical conditions. However, to treat COVID-19 in hospitalized patients the rapid increase circulating serum concentrations of

25(OH)D₃ might help reduce risk of morbidity and mortality in COVID 19 patients with a history of other chronic disorders (37).

Of note, up to now there is no standardized practice and/or cost benefit of using oral 25(OH)D₃ compared to oral vitamin D₃ (14). Based on the market prices in Italy in 2019, the cost per IU of 25(OH)D₃ could be about six times lower than that of cholecalciferol (17). Based on these findings, we strongly recommend further RCTs to consider 25(OH)D₃ as an alternative vitamin D supplementation in vitamin D deficient or insufficient adults with an acute medical condition like COVID-19.

Although the experimental design of the study was randomized, double-blind, and placebo-controlled with a high adherence rate are major strengths of this study; this study also had several weaknesses and limitations. This study was a pilot study that evaluated biochemical and clinical outcomes in patients with COVID-19 who received orally daily 25µg of 25(OH)D₃. This dose which is equivalent to ingesting 3000-6000 IU/day of a vitamin D₃ supplement would be considered to be 2-3 fold higher than what is recommended by the Endocrine Society Guidelines on Vitamin D for adults which is 1500-2000 IUs daily (13). The analysis of this pilot study showed that this dose of 25(OH)D₃ only had effect on decreasing the NLR that has been related to improved clinical outcomes (30, 31). Although we did not observe that the significant decrease in the NLR in the patients who ingested 25(OH)D₃ was related to improve clinical outcomes compared to the control group, this could be due to the need to more rapidly improved serum 25(OH)D concentrations by giving a higher dose of 25(OH)D₃ as has been recently reported (9, 34, 38). This pilot study was performed on 53 patients in each group. It was underpowered for detecting significant differences in clinical outcome measures. RCTs with a larger sample size and with higher dose of 25(OH)D₃ is needed to confirm the potential effect of 25(OH)D₃ on reducing clinical outcomes in COVID-19 patients.

Conclusion

Our findings indicate that using 25 µg oral 25(OH)D₃ daily is safe and effective in increasing and maintaining optimal 25(OH)D₃ serum concentrations in adults with COVID 19. Treatment with oral 25(OH)D₃ has a potential benefit in improving immune function by increasing lymphocyte percentage and decrease the RCT in hospitalized patient with COVID-19. Indeed, our findings showed 25(OH)D₃ intervention significantly decreased the neutrophil-to-lymphocyte ratio in the COVID-19 patients that was associated with improved clinical outcomes.

The ability to rapidly raise and sustain circulating levels of 25(OH)D₃ will result in the immediate availability of 25(OH)D₃ that can be quickly converted to the immunomodulatory hormone 1,25(OH)₂D₃. Therefore, there is a strong rationale to consider using 25(OH)D₃ to improve the patient's vitamin D status, to help maximize their immune system and fight the COVID-19 pandemic.

Acknowledgements

We appreciate of all health providers, and laboratory staffs of Ziaian Hospital, Shariati Hospital and Shohadaye Pakdasht Hospital. Dishman Carbogen Amcis Ltd as a partner of BU manufactured and supplied both the active, from its subsidiary location in the Netherlands, and the finished dosage form from its facility in Ahmedabad India, for the trials.

Arpit J Vyas Global Managing Director of DCAL commented “we are proud to have been able to support this investigation that could facilitate the global fight against Covid -19. Our collaboration with BU and Dr Holick continues to demonstrate the remarkable properties of Vitamin D analogues and their potential role in treating many unmet clinical needs”

Funding

This work was supported by Tehran University of Medical Sciences (grant NO.: 47095-235-1-99).

Declaration of interests

The authors declare the following personal relationships which may be considered as potential competing interests:

Michael F. Holick was a consultant for Quest Diagnostics Inc. is a consultant for Ontometrics Inc, Biogen Inc., received a grant from Carbogen Amcis BV and was on the speaker's Bureau for Abbott Inc.

The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Submission declaration and verification

The work described has not been published previously, that it is not under consideration for publication elsewhere. Its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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Table 1: Demographic characteristics and biochemical tests at the base line

Baseline characteristics	N: 25(OH)D/placebo	25(OH)D	Placebo	p-value
Age (years)	53/53	50±15	49±13	0.6
Sex (female)	53/53	41%(22)	38%(20)	0.7
Spo2 (%)	52/52	90±5	89±7	0.6
Heart rate (NO/min)	50/49	89±11	88±15	0.8
Respiratory rate (NO/min)	51/49	19.5±3	20±4.5	0.4
Temperature (o C)	51/48	37±0.9	37±0.6	0.7
Systolic BP (mmHg)	51/49	117±16	120±16	0.4
Diastolic BP (mmHg)	51/49	73±11	75±10	0.4
chronic disorder	53/53	55%(29)	45%(24)	0.3
Smoking	50/49	12%(6)	9%(4)	0.6
CT involvement - Bilateral	42/39	88%(37)	89%(35)	0.3
CT severity	37/33			
Moderate & sever		65%(24)	73%(24)	0.5
None & mild		35%(13)	27%(9)	
BMI (kg/m ²)	53/53	29±6	29±5.5	0.8
Hematology				
W.B.C (*1000C/ml)	53/52	6.9±3.3	7.4±3.9	0.6
R.B.C (Mil C/ml)	53/53	4.6±0.7	4.6±0.8	0.5
Hemoglobin (g/dl)				
M.C.V (fl)	53/53	84±7.5	85±8	0.6
Platelet (*1000 C/ml)	53/53	202±85	213±104	0.6
Neutrophils (%)	52/53	71±13	73±12	0.6
Lymphocyte (%)	52/53	25±12	23±11	0.4
Blood Biochemistry				

25(OH)D (ng/ml)	53/53	19±8	18±8	0.7
Ln.ESR.1Hr (mm/hr)	48/45	3.5±1.0	3.5±0.6	0.6
Ln. BUN (mg/dl)	53/51	3.3±0.5	3.4±0.5	0.6
Cr (mg/dl)	53/51	1.1±0.3	1.0±0.3	0.4
Ln. AST (U/L)	35/33	3.4±0.5	3.63±0.6	0.1
Ln. ALT (U/L)	35/33	3.5±0.5	3.64±0.6	0.4
ALP (U/L)	33/31	140 (110)	133 (146)	0.1
P (mg/dl)	30/28	3.6±0.7	3.8±0.6	0.4
Ca (mg/dl)	34/32	8.7±0.5	8.6±0.6	0.3
Na (mEq/lit)	51/51	137±4	138±4	0.2
K (mEq/lit)	51/52	4.2±0.5	4.2±0.5	0.9
Mg (mg/dl)	37/30	2.0±0.2	2.1±0.3	0.6
Alb (gr/dl)	31/27	4.4±0.4	4.0±0.7	0.02
Bilirubin Total (mg/dl)	33/31	0.7 (0.6)	0.9 (0.5)	0.7
Bilirubin Direct (mg/dl)	32/31	0.2 (0.1)	0.2 (0.2)	0.8
Ln. CPK (U/lit)	37/31	4.8±0.8	4.8±0.8	0.8
Ln. LDH (U/lit)	44/42	6.2±0.5	6.3±0.5	0.3
V.B.G				
PH	32/27	7.4±0.1	7.4±0.1	0.2
PCo2 (mmHg)	32/26	40.5±7	39±7	0.4
Po2 (mmHg)	31/26	34±15	34±10	0.9
HCo3 (mmol/L)	32/27	24±3	24±3	0.9
Serology				
CRP (Qual)	43/44			
negative		32.6%(14)	27.3%(12)	0.4
+1		27.9%(12)	31.8%(14)	
+2		18.6%(8)	29.5%(13)	

+3		20.9%(9)	11.4%(5)	
<p>Numerical variables were expressed as the mean \pm SD for parametric tests or median (IRQ) for non-parametric tests and categorical variables were presented as percentages. N=available data for each variable</p> <p>Albumin (Alb), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), Body mass index (BMI), calcium (Ca), creatinine (Cr), creatine phosphokinase (CPK), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), bicarbonate (HCO₃), potassium (K), lactate dehydrogenase (LDH), magnesium (Mg), sodium (Na), phosphorus (P), partial pressure of oxygen (PO₂), partial pressure of carbon dioxide (PCO₂), VBG Venous Blood gases (V.B.G)</p>				

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Table 2: Prognostic factors for COVID-19 at baseline

Baseline characteristics	N (25(OH)D ₃ /placebo)	25(OH)D ₃	Placebo	p-value
Age \geq 65 (years)	53/53	13%(7)	13%(7)	1.0
Disease severity (based on CDC criteria)	53/53	68%(36)	60%(32)	0.4
History of Chronic disorders				
Hypertension	53/53	34%(18)	28%(15)	0.5
Cardiac disorder	53/53	9%(5)	15%(8)	0.4
Diabetes mellitus	53/53	26%(14)	21%(11)	0.5
Immunological	53/53	4%(2)	0	0.5*
Liver	53/53	1.9%(1)	0	1.0*
Renal	53/53	4%(2)	2%(1)	1.0*
Neurological	53/53	4%(2)	0%	0.5*
Lung	53/53	7.5%(4)	13%(7)	0.3
Lymphocytes < 800	52/53	19%(10)	22%(12)	0.6
At baseline, there was no significant difference in history of chronic disorders including hypertension, cardiovascular disorder, diabetes mellitus, lung, liver and kidney diseases and neurological and immunological disorders. N=available data for each variable. *Fisher Exact Test				

Table 3: clinical and biochemical outcomes

Clinical outcomes	N (25(OH)D3/placebo)	25(OH)D3	Placebo	p-value
Hospitalization day	53/53	5 (3)	6(5.5)	0.1
Death	53/53	6%(3)	9%(5)	0.7*
Oxygen therapy	53/53	60%(32)	64%(34)	0.7
Intubation	53/53	4%(2)	9%(5)	0.4*
Ventilator	53/53	4%(2)	9%(5)	0.4*
ICU admitted	53/53	11%(6)	19%(10)	0.3
ICU (days) (range)	53/53	7 (0-7)	11 (0-11)	0.2
Biochemical outcome				
Mean diff WBC(*10 ³)	53/52	0.1±3.1	1.8±4.2	0.02
Mean diff Lymphocyte(*10 ³)	47/51	2.8±12.3	-2.7 ±11.9	0.02
Mean difference LDH (U/lit)	27/18	-5 (177)	-41.5 (221)	0.6
Mean difference Neutrophil(*10 ³)	47/51	-0.2 (0.8)	0.23 (1.1)	0.01
Mean difference Platelet (*10 ³)	53/50	29 (83.5)	21 (65)	0.6
NLR at date of bassline	52/52	4.2 ±3.8	3.4 ±1.6	0.3
NLR at date of release	48/52	3.3 ±2.5	5.3±4.8	0.02
Treatments				
Antiviral therapy	53/53	4% (2)	5%(3)	1
Corticosteroids therapy	53/53	40% (21)	53% (28)	0.24
*Fisher Exact Test				
lactate dehydrogenase (LDH), Neutrophil to lymphocyte ratio (NLR)				

Figure Legends

Figure 1: Flowchart of participants through the study

Figure 2. Alterations in serum concentrations of 25(OH)D in the 25(OH)D3 and placebo groups

The serum levels of 25(OH)D were significantly increased in patients who received 25(OH)D3 compared to the placebo group. After 30 days of ingesting 25(OH)D3 or placebo, circulating concentrations of 25(OH)D were significantly increased in the patients who received 25(OH)D3 (N=34) compared to the placebo group (N=24). Treatment group 42.0 ± 2.3 ng/ml, vs. placebo: 19.3 ± 1.7 ng/ml. After 60 days the 24 patients in the treatment group had a serum concentration of 25(OH)D of 59.6 ± 3.8 ng/ml, compared to 19 patients in the placebo group who had a serum concentration of 25(OH)D of 19.4 ± 1.6 ng/ml.; respectively.

The error bars are mean \pm SE; * P <0.001

Figure 3: The neutrophil-to-lymphocyte ratio (NLR) in the 25(OH)D3 and placebo groups at the time of hospitalization and after release date

The neutrophil-to-lymphocyte ratio (NLR) at the time of discharge was significantly decreased in patients who received 25(OH)D3 compared to the placebo group. After the patients were released from the hospital, they had a follow-up visit 1 and 2 months later at which time the NLR in their circulation was determined. The circulating NLR decreased in both groups and were statistically no different.

The NLR at the first-month follow-up in patients who received 25(OH)D3 (N=33) and placebo (N=28) was 1.7 ± 0.2 , vs. 1.8 ± 0.2 , respectively.

After 2 months follow-up, the NLR in the treatment group (N=32) and placebo group (N=22) was 1.9 ± 0.3 vs. 1.9 ± 0.2 , respectively.

The error bars are mean \pm SE; * P <0.05

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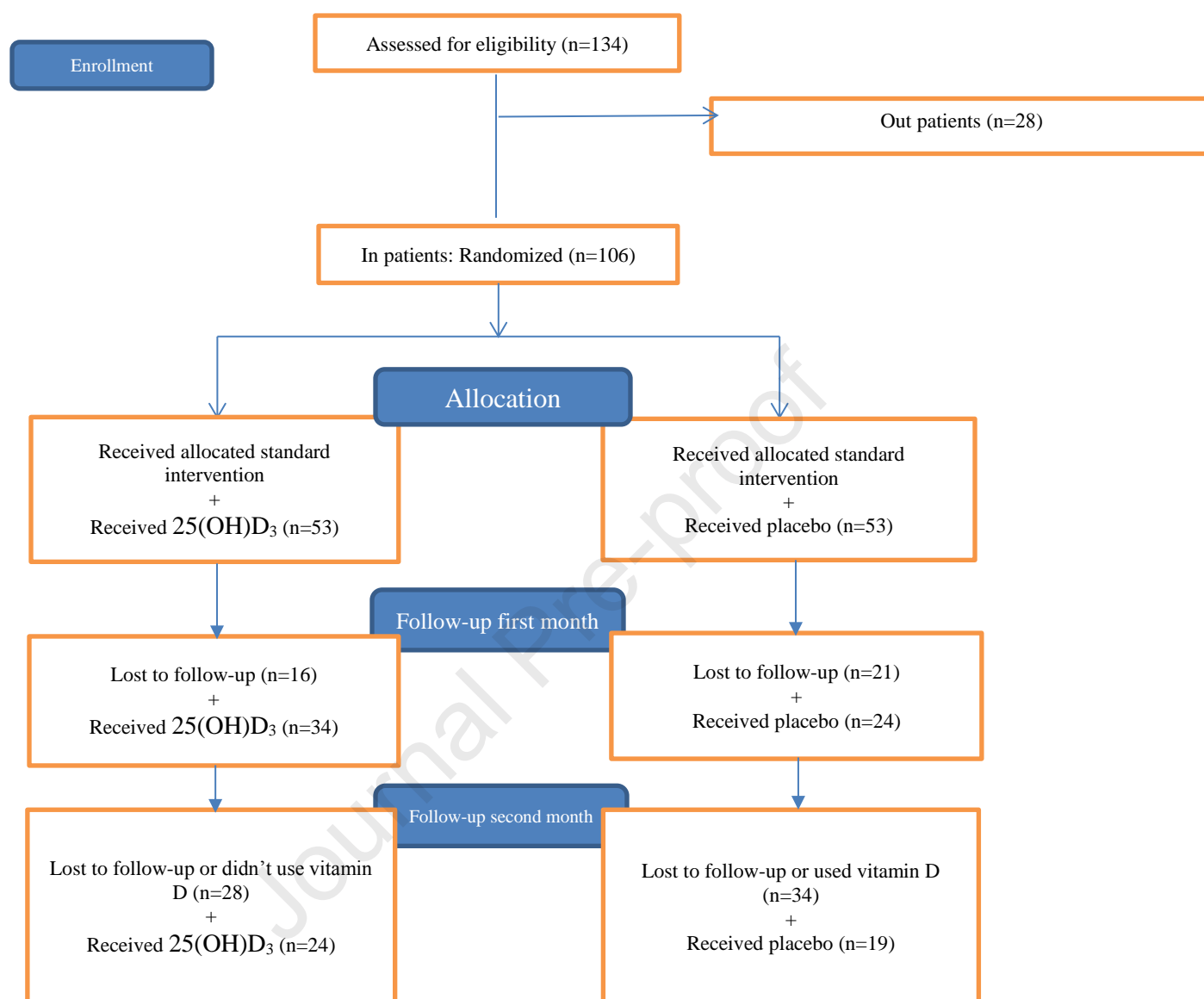
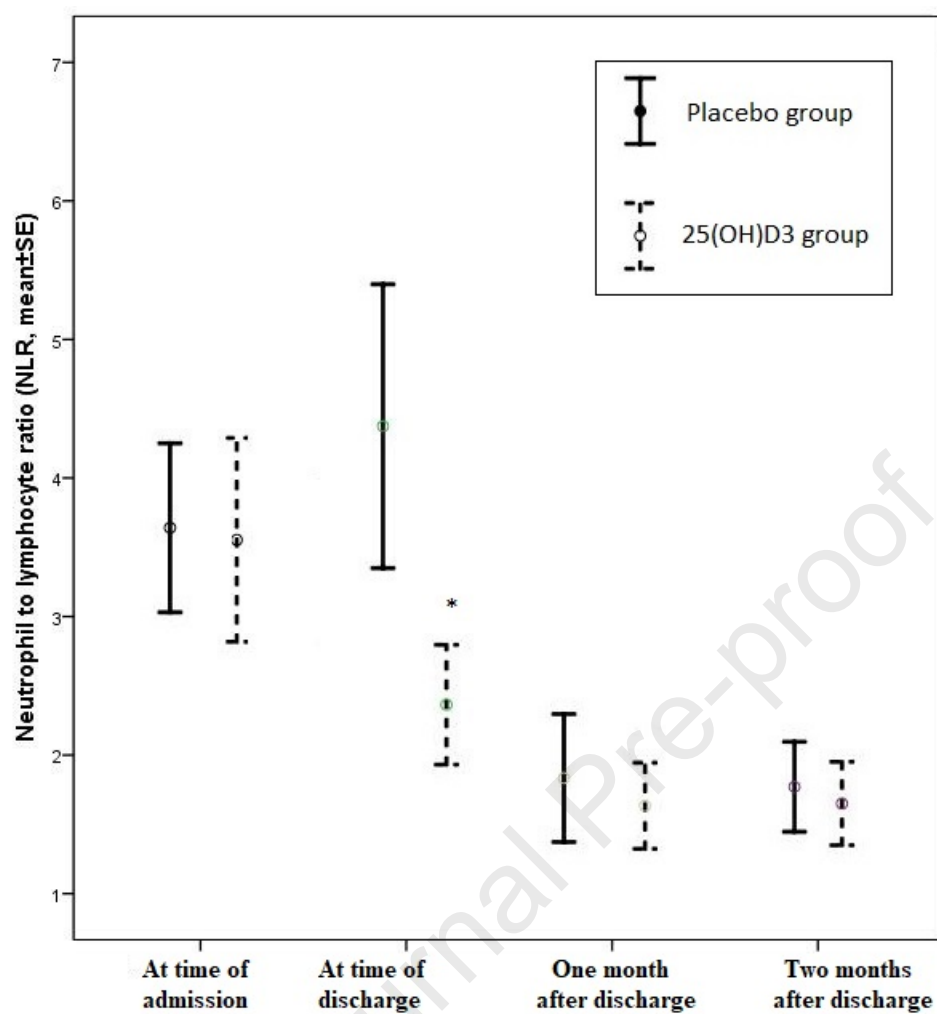
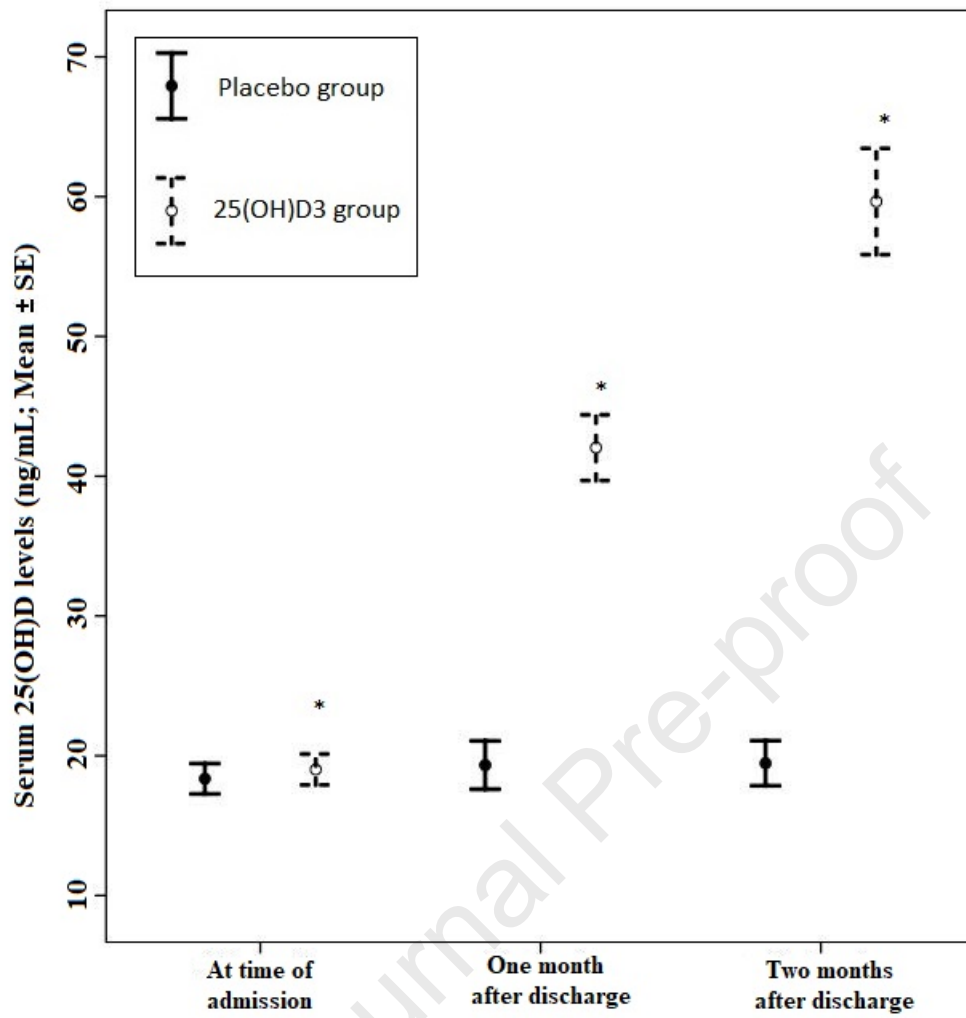


Figure 1: Flowchart of participants through the study





Highlights:

- 25(OH)D₃ intervention decreased the neutrophil-to-lymphocyte ratio (NLR) in the COVID-19 patients
- The lower NLR was significantly associated with reduced ICU admission days and mortality.
- After two months of taking 25(OH)D₃ or placebo, all patients who received 25(OH)D₃ became sufficient (>30ng/ml).

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The authors declare the following personal relationships which may be considered as potential competing interests:

Michael F. Holick was a consultant for Quest Diagnostics Inc. is a consultant for Ontometrics Inc and Biogen Inc., received a grant from Carbogen Amcis BV and was on the speaker's Bureau for Abbott Inc. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.